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Multi-level toxicity assessment of polylactic acid (PLA) microplastics on the cladoceran *Daphnia magna*

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ABSTRACT

The accumulation of plastics waste in the environment has raised a worrisome concern, moving the society to seek out for sustainable solutions, such as the transition from the use of fossil-based, conventional plastics to bioplastics (BPs). However, once in the environment bioplastics have the same probability to accumulate and experience weathering processes than conventional plastics, leading to the formation of microplastics (MPs). However, to date the information on the potential toxicity of MPs originated from the weathering of bioplastics is limited. Thus, this study aimed at investigating the adverse effects induced by the exposure to MPs made of a bioplastic polymer, the polylactic acid (PLA), towards the freshwater cladoceran *Daphnia magna*. Organisms were exposed for 21 days to three concentrations (0.125 μg/mL, 1.25 μg/mL and 12.5 μg/mL) of PLA microplastics (hereafter PLA-MPs). A multi-level approach was performed to investigate the potential effects through the biological hierarchy, starting from the sub-individual up to the individual level. At the sub-individual level, changes in the oxidative status (i.e., the amount of reactive oxygen species and the activity of antioxidant and detoxifying enzymes) and oxidative damage (i.e., lipid peroxidation) were explored. Moreover, the total caloric content as well as the content of protein, carbohydrate and lipid content assess were used to investigate the effects on energy reserves. At individual level the changes in swimming activity (i.e., distance moved and swimming speed) were assessed. Our results showed that the exposure to PLA-MPs induced a slight modulation in the oxidative status and energy reserves, leading to an increase in swimming behavior of treated individuals compared to control conspecifics. These results suggest that the exposure to MPs made of a bioplastic polymer can induce adverse effects similar to those caused by conventional polymers.

1. Introduction

Plastic pollution represents one of the main environmental issues that our society have currently to face (Shen et al., 2019), indeed the persistence and the inappropriate disposal of plastic objects and materials at their end-life have raised the worrisome problem of plastic contamination of natural and anthropic ecosystems (Li et al., 2020; Rai et al., 2021). The degradation and fragmentation of large-sized plastic items in the environment have been identified as the main sources of microplastics (MPs, i.e., any plastic items ranging between 1 μm and 1 mm in size; Hartmann et al., 2019) to marine, freshwater and terrestrial ecosystems. Nowadays, MPs are ubiquitous in ecosystems worldwide (Thacharodi et al., 2024; Su et al., 2022). Although soils, freshwaters and marine environments are considered as the main sinks for MPs (Xiang et al., 2022; Koutnik et al., 2021), several studies highlighted their presence also in remote areas, such as glaciers (Ambrosini et al., 2019; Crosta et al., 2022) and Polar regions (Mishra et al., 2021). To tackle the environmental issue related to plastic (and MP) pollution, different strategies were implemented, including the improvement of the recycling processes (Gazzotti et al., 2022; Vollmer et al., 2020), the ban of MPs from personal care products and, the transition from the use of conventional, fossil-based polymers to bioplastics (European Bioplastics, 2023; Nandakumar et al., 2021; Shah et al., 2021).

Bioplastics materials can be defined as bio-based, biodegradable, or both (European Bioplastics, 2023). These materials have the same or almost the same characteristic as conventional plastics. In addition, bioplastics present some environmentally relevant benefits, such as a reduction of the carbon footprint or more waste management options (European Bioplastics, 2023). In 2022, the global production of bioplastics accounted for 2.23 million tons, corresponding to the 1 % of the

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annual plastic production only (European Bioplastics, 2023). However, it has been predicted a 36 % increase in bioplastics use in the next years, reaching 3.5 million in 2027, because of a notable development of polymers, such as polylactic acid and the polyhydroxyalkanoates (European Bioplastics, 2023). According to the increase in bioplastics use, a great amount of bioplastics might enter and accumulate the environment without a proper management. In fact, although bioplastics objects should completely biodegrade within six months in an industrial composting facility (European Bioplastics, 2023), a slower rate of degradation (up to 3–5 years) was reported in the environment (Ncube et al., 2020). Moreover, considering the current trend in waste disposal and waste mismanagement, it can be supposed that bioplastic objects have the same potential to become trash, to end up in the environments and to experience degradation, resulting in the formation of MPs as for conventional plastics (Shruti and Kutralam-Muniasamy, 2019). Among bioplastics, one of the most promising materials to be used as an alternative to fossil-based plastics is the polylactic acid (PLA). PLA is a bio-based and biodegradable plastics derived from fermentative lactic acid (Ahmad et al., 2022) commonly used in food packaging and shopping bags, cutlery and mulch films (Ncube et al., 2020). Some studies revealed that, once in environment, PLA can suffer degradation and generate MPs, which can therefore represent a threat for organisms similarly to their fossil-based counterpart (Ainali et al., 2022; Venâncio et al., 2022; Ge et al., 2021; Shruti and Kutralam-Muniasamy 2019).

The ingestion of MPs from both conventional and bioplastic polymers has been confirmed for aquatic and terrestrial organisms (Wang et al., 2022; Magara et al., 2019; De Felice et al., 2020, 2019). After the ingestion, fossil-based MPs can induce a large variety of adverse effects, such as the onset of oxidative stress (Ainali et al., 2022; Parolini et al., 2020a Parolini et al., 2020b) and inflammatory conditions (Duan et al., 2022), changes in biometric traits and growth rate (De Felice et al., 2018; Green, 2016), as well as in swimming behavior and reproduction (de Oliveira et al., 2021; De Felice et al., 2019). Despite these findings, little is still known about the effects induce by the exposure and ingestion of MPs made of bioplastics towards aquatic organisms. A first study has shown that the exposure to irregular shaped PLA-MPs (3.4 μm in size; 19.6 μg/L) did not affect the feeding activity, the body size and the population growth of *D. magna* (Gerdes et al., 2018). In contrast, another investigation has highlighted that the exposure to PLA-MPs (5.13×10^7 particle/L) negatively affected the reproduction of the same model species (Zimmermann et al., 2020). de Oliveira et al. (2021) have observed a reduction in the swimming distance and speed of *Danio rerio* larvae after 5 days of exposure to PLA-MPs (3 and 9 mg/L). Moreover, a recent comparative study has highlighted that the exposure to the fossil-based polymer polyethylene terephthalate (PET) and PLA-MPs returned similar adverse effects on the fertilization success of the solitary ascidian (*Microcosmus exasperates*; Anderson and Shenkar, 2021). In addition, as these polymers are biodegradable, their toxicity can occur as the consequence of the release of degradation by-products, which can be produced within the organisms during the transit or digestion processes (e.g., Duan et al., 2024; Parolini et al., 2024). Although these findings suggest the potential hazard of PLA-MPs towards aquatic organisms, they are often limited to a single or few endpoints and returned contrasting results.

Polylactic acid was selected as focal polymer of the present study for two reasons. First, the most of bioplastics object on the market are currently made of PLA (Plastics Europe, 2023) and second, despite the results of some recent studies, there is still a dearth of information regarding the hazard of MPs made of this polymer towards organisms and ecosystems. Thus, the aim of this study was to investigate the potential toxicity induced by the exposure to three concentrations (0.125 μg/mL, 1.25 μg/mL and 12.5 μg/mL) of PLA-MPs towards the freshwater cladoceran *Daphnia magna* through the application of a multi-level approach.

As several studies have confirmed that the exposure to MPs made of diverse polymers can cause an oxidative stress condition in aquatic organisms (e.g., Ainali et al., 2022;Parolini et al., 2020aParolini et al., 2020b), a battery of oxidative stress-related biomarkers was applied. Specifically, changes in the amount of reactive oxygen species (ROS), activity of antioxidant (superoxide dismutase - SOD; catalase - CAT and gluthatione peroxidase - GPx) and detoxifying (glutathione S-transferase - GST) enzymes, and lipid peroxidation were measured. Then, as the activation of the antioxidant machinery is an energy demanding activity (e.g., Resende et al., 2022), the protein, carbohydrate and lipid content, as well as the total caloric content, was measured to assess modulations in energy resources. Lastly, as organisms suffering an oxidative stress condition and an alteration in energy reserves can experience alterations in the swimming behavior (Félix et al., 2023; Chen et al., 2020), changes in swimming behavior were investigated through a video-tracking analysis, considering the distance moved and the swimming speed as endpoints.

2. Materials and methods

2.1. Preparation and characterization of PLA-MPs

PLA-MPs used in this study were obtained from commercial PLA chips after several cycle of grinding and freezing in liquid nitrogen (Parolini et al., 2020a; Parolini et al., 2020a). At the end of this process to obtain particles in the range of MPs, the particles were sieved with a sieve having 1 mm of mesh size (Hartmann et al., 2019). The obtained MPs were elongated and had an irregular shape (Fig. 1A) with a mean length of 164 ± 0.005 μm ($n = 500$ particles). PLA-MPs were grouped in four classes of size: the 20 % of the MPs had a diameter smaller than 50 μm, the 28.42 % had a diameter ranging between 50 μm and 100 μm, while the diameter of the 33.68 % of items ranged between 100 μm and 250 μm and the diameter of the 17.9 % ranged between 250 μm and 1 mm. The polymeric composition of PLA-MPs was confirmed by using a Fourier Transformed Infrared Spectroscope (FTIR) Perkin Elmer Spectrum 100. In detail, the spectrum showed the diagnostic signals that are the CH_3 stretching bands at 3000 cm^{-1}, the ester C═O stretching band at 1757 cm^{-1}, the CH and CH_3 deformation bands between 1450 and 1350 cm^{-1} and the C—O–C stretching bands between 1250 and 1000 cm^{-1} (Fig. 1B). Overall, the PLA-MPs showed a preserved chemical integrity, confirming that the freezing-grinding process did not have effects on the structure of the material.

2.2. Exposure to PLA-MPs

Daphnia magna organisms used in this experiment came from the husbandry located in the laboratory of the University of Milan. The husbandry was maintained following the condition previously described by De Felice and co-authors (2022). In detail, 30 individuals were maintained in glass beaker (30 individuals/L) filled with San Benedetto® commercial water (conductivity 415 μmS cm^{-1} at 20 °C, pH 7.42, 301 mg/L HCO_3^-, 48.6 mg/L Ca^{2+}, 28.2 mg/L Mg^{2+}). Individuals were fed ad libitum three times a week with the yeast *Saccharomyces cerevisiae* (0.5 g/L) and a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells/individual/day up to 8-days old individuals, 16×10^6 cells/individual/day for individuals older than 8 days) (De Felice et al., 2022). The husbandry was maintained in a thermostatic chamber at 20.0 ± 0.5 °C under a 16 h light:8 h dark photoperiod. The organisms used for the experiments were born from the same mothers and randomly selected to be included in control or treated groups. As no information on the environmental concentrations of PLA-MPs is available, a preliminary acute toxicity test was performed following the OECD Guidelines for the Testing of Chemicals (i.e., *Daphnia* sp. Acute Immobilisation Test n. 202; OECD, 2004). Daphnids <24 h were exposed to a wide range (i.e., 0.05, 0.1, 0.5, 1, 2, 5, 7, 10 L and 15 μg/mL) of PLA-MPs concentrations for 48 h. The selected concentrations were in the same range of previous experiments performed to assess acute toxicity of PLA and PBAT microplastics on zebrafish (i.e.,

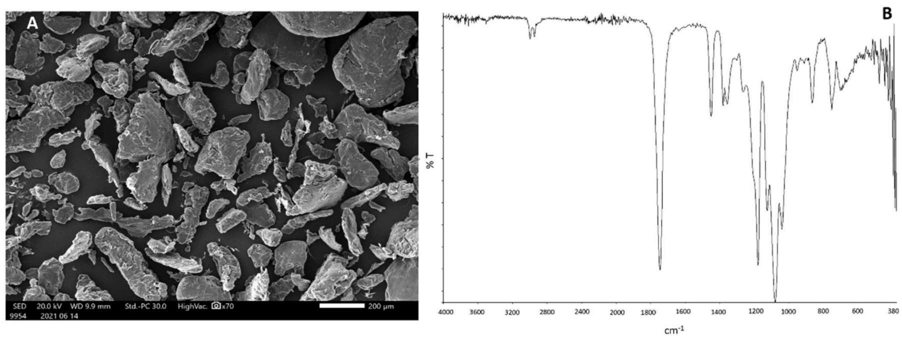

Fig. 1. Scanning electron microscopy (SEM) image (A) and Fourier Transformed Infrared Spectroscope (FT-IR) spectrum (B) of micronized polylactic acid microplastics (PLA-MPs).

0, 1, 2, 4, 8, 16 and 32 mg/L; Zhang et al., 2024), PLA microplastics (0.5, 1, 2.5, 5, 7.5, 10, and 20 mg/L; Luangrath et al., 2024) and polystyrene nanoplastics on *Daphnia magna* (0.5, 1, 2, 5, 7, and 10 mg/L; De Felice et al., 2022). For each tested concentration and the control, five replicates with five daphnids (i.e., neonates <24 h old) each were used. Immobilization was recorded after 24 and 48 h, to determine the EC_{50}. As none of the concentrations tested during the implementation of the acute toxicity test induced the immobilization of all the exposed organisms neither after 24- nor 48 h of exposure, the EC_{50} value could not calculated. Thus, the concentrations used for the sub-lethal exposures were selected to match those used in previous studies aimed at investigating the toxicity of MPs on *D. magna* (Parolini et al., 2023; De Felice et al., 2019). Organisms were exposed to three different PLA-MPs concentrations (0.125 μg/mL, 1.25 μg/mL and 12.5 μg/mL) for 21 days following the procedure described by De Felice and co-authors (2022). Three different, independent exposures, lasting 7, 14 and 21 days, were performed under semi-static renewal conditions. For each experimental group, three independent replicates were prepared in 100 mL beakers including 20 daphnids (neonates < 24 h old), for a total of 60 individuals per treatment and time point. Individuals from all the experimental groups were fed every other day and they were maintained at the same condition described above until the end of the experiment. At the end of each exposure (i.e., after 7, 14 and 21 days) thirty organisms per treatment (i.e., 10 individuals per replicate) were randomly selected from the beakers and their swimming activity was recorded (see Section 2.4). After completing behavioral analyzes, for each time point, all the alive individuals from each beaker were pooled in an Eppendorf tube, frozen in liquid nitrogen and placed at −80 °C until biomarker analyzes (see Section 2.3).

2.3. Oxidative stress and energetic biomarkers

Biomarkers were applied on pools composed by 18–20 individuals, one from each of the three independent replicates per treatment, according to the procedures described elsewhere (De Felice et al., 2020, 2022). *D. magna* individuals were homogenized in 100 mM potassium phosphate buffer (100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitors 1:100 v/v; pH 7.4) with a motor pestle. The obtained homogenates were centrifuged at 15,000 × *g* for 20 min at 4 °C and the supernatant was then processed to assess oxidative stress (i.e., ROS content, activity of SOD, CAT, GPx and GST, and lipid peroxidation) and energetic (i.e., protein, carbohydrate and lipid content) biomarkers. Concerning oxidative stress biomarkers, SOD activity was calculated by measuring the reduction of the inhibition of cytochrome c (10 μM) due to O_2^- generated from the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 mM) reaction for 1 min at λ = 550 nm. CAT activity was assessed measuring the depletion of H_2O_2 (50 mM) for 1 min at λ = 240 nm. The GPx activity was assessed through the measurement of the depletion of NADPH (0.12 mM) during a reaction with H_2O_2 (0.2 mM), glutathione (2 mM), sodium azide (1 mM) and glutathione reductase (2 U/mL) for 1 min at λ = 340 nm. The glutathione S-transferase (GST) activity was evaluated by measuring the conjugation of the glutathione (1 mM) to the 1-chloro-2,4 dinitrobenzene (CDNB; 1 mM) for 1 min at λ = 340 nm. All the enzyme activities were normalized on the protein content, which was measured according to the Bradford method (Bradford, 1976). Lipid peroxidation was measured following the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979). Concerning energetic biomarkers, the protein content was measured through the Bradford method as reported above. The total carbohydrate content was calculated through the anthrone reagent method (Jermyn, 1975). The sulfo-phospho-vanillin method (Frings et al., 1972) was used to measure the lipid content. Protein, carbohydrate and lipid content was normalized on the number of processed individuals. The caloric content of *D. magna* was calculated according to Sancho and co-authors (2009), assuming 4.10 cal/mg for carbohydrates, 5.65 cal/mg for total proteins and 9.45 cal/mg for lipids. Results were expressed as mcal/individual. All the methods described above relied of spectrophotometric procedures and the readings were performed using a Genova Bio spectrophotometer (Jenway).

2.4. Video-tracking analysis

The changes in swimming activity induced by the exposure to PLA-MPs were assessed through a video-tracking system following a protocol previously described by De Felice and co-authors (2022). In detail, for each treatment, the swimming activity of 30 individuals was recorded, and then tracked, at the end of 7, 14 and 21 days of exposure. Each single individual was transferred into an "arena", which is a well of a 12-well plate (11.5 cm × 8 cm × 1.5 cm) filled with 3 mL of the culture medium. The individuals were left to acclimatize for 5 min. The swimming activity of each individual was then recorder for 30 s using an iPhone 6. The obtained 1080p Full HD (30 frames/s) videos were analyzed using the ImageJ plugin AnimalTrack (Gulyas et al., 2016). A couple of coordinates were assigned to each individual for each frame. The "tracker module" and "the analyze module" were used to calculate the distance moved (expressed in mm) and the swimming speed (cm/*sec*) of all the individuals.

2.5. Statistical analysis

Normality and homoscedasticity of data were preliminarily checked through the application of the Shapiro-Wilk normality test and studentized Breusch-Pagan test, respectively. When data did not fit a normal distribution, they were log transformed. Data of SOD, CAT, protein and lipid content were log normalized because they did not fit normal distribution. After preliminary checking, the effects of PLA-MPs, time of exposure and their interaction on oxidative stress and energetic biomarkers were analyzed by the application of a two-way Analysis of the Variance (ANOVA), considering each biochemical endpoint as the dependent variable, while the treatment and the time of exposure as predictors. The effect of treatment, time of exposure and their interaction on behavioral endpoints was investigated by Linear Mixed Models (LMM). Treatment and time of exposure were included in the models as fixed effect factors, while the exposure tank (i.e., experimental replicate) was included as a random factor. A Tukey' HSD post-hoc test was carried out to identify significant differences between treatments. Significance was set to be with $P < 0.05$ (*) and $P < 0.01$ (**). All the analyzes were run in R 4.03 (R Core Team 2020).

3. Results

After 7 days of exposure, a slight mortality was noted in individuals from the control (1.52 %) and 1.25 μg/mL (1.56 %), while no individual died in the other two experimental groups. After 14 days of exposure, the mortality accounted to 7.94 % in the control group, while to 3.08 %, 6.35 % and 1.59 % in the 0.125 μg/mL, 1.25 μg/mL and 12.5 μg/mL experimental groups, respectively. After 21 days of exposure, the mortality accounted to 3.08 % in the control group, while to 6.35 %, 6.45 % and 13.33 % in the 0.125 μg/mL, 1.25 μg/mL and 12.5 μg/mL experimental groups, respectively.

In addition, microscopy analyzes performed after the administration of a very high concentration (50 μg/mL) of PLA-MPs allowed to highlight their transit in the digestive tract of both neonates (< 24 h old) and adults (21 days old) and to confirm the capability of *D. magna* individuals to ingest PLA-MPs at both neonatal and adult stage (Fig. 2).

3.1. Response of oxidative stress-related biomarkers

The complete outcome of the statistical analyzes performed on data of oxidative stress biomarkers is reported in Supporting Information (Table S1).

No effect of the treatment ($F_{3,20} = 0.787$; $P = 0.515$), the exposure time ($F_{2,20} = 0.713$; $P = 0.502$) and the time × treatment interaction ($F_{6,20} = 1.987$; $P = 0.115$) was found on ROS content (Fig. 3 a).

A significant effect of the exposure time ($F_{3,22} = 8.880$; $P = 0.001$) was noted on SOD, showing an activity increase in 7-days old ($P = 0.005$) and 14-days old ($P = 0.003$) individuals compared to 21-days old ones, independently of the treatment. Although no effect of the treatment was noted ($F_{2,22} = 1.460$; $P = 0.252$), a significant time × treatment interaction ($F_{6,22} = 4.335$; $P = 0.005$; Fig. 3b) occurred. Specifically, the activity measured in 14-days old individuals exposed to 0.125 μg/mL PLA-MPs was significantly lower compared to conspecifics from the corresponding control ($P = 0.025$).

A significant effect of the exposure time ($F_{2,23} = 65.779$; $P < 0.001$) and the treatment ($F_{3,23} = 3.762$; $P = 0.024$) was noted on CAT activity, while no effect was observed for the interaction of the main predictors ($F_{6,23} = 1.884$; $P = 0.126$). CAT activity was significantly lower in 21-days old individuals compared to 7- and 14-days old ones ($P < 0.001$ in both the cases), independently of the treatment. Moreover, the activity measured in individuals exposed to 12.5 μg/mL was significantly higher compared to those from the control group ($P = 0.030$; Fig. 3c).

A significant effect of the exposure time ($F_{2,24} = 3.688$; $P = 0.040$) and the treatment ($F_{3,24} = 15.285$; $P < 0.001$) was noted on GPx. The activity measured in 21-days old individuals was significantly lower compared to 14-days old conspecifics ($P = 0.050$), while a significant activation was noted in individuals exposed to the highest tested concentration compared to the control group ($P < 0.001$). Moreover, a significant time × treatment interaction ($F_{6,24} = 6.730$; $P < 0.001$) occurred, with the activity measured in 14-days old individuals exposed to 1.25 μg/mL and 12.5 μg/mL of PLA-MPs that was significantly higher compared to individuals from the corresponding control ($P < 0.001$ in both the cases; Fig. 3d). In addition, also the activity measured in 21-days old individuals exposed to the highest tested concentration was significant higher compared to control ($P = 0.003$; Fig. 3d).

Although no effect of the exposure time ($F_{2,24} = 0.383$; $P = 0.685$) was noted, the treatment induced a significant modulation of GST ($F_{3,24} = 8.093$; $P < 0.001$). The activity measured in individuals from the 12.5 μg/mL treatment group was significantly lower compared to conspecifics from the control group ($P = 0.020$; Fig. 3e), independently of the exposure time. Moreover, a significant time × treatment interaction ($F_{6,24} = 7.531$; $P < 0.001$) was noted, with an activity increase observed in 7-days old individuals exposed to 1.25 μg/mL of PLA-MPs compared to the corresponding control ($P = 0.025$). The activity measured in 21-days old specimens exposed to 1.25 μg/mL of PLA-MPs was significantly

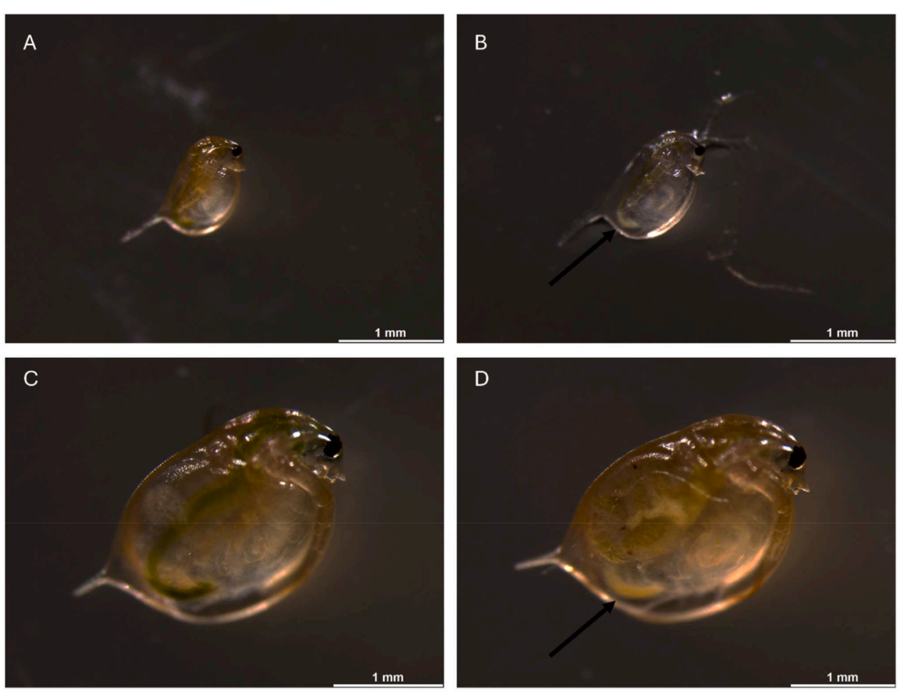

Fig. 2. Picture of a daphnid (i.e., a neonate <24 h old; A) and an adult (21 days old; C) from the control group and a daphnid and an adult from the 12.5 μg/mL of PLA-MPs exposure group. The black arrows indicate the presence of PLA-MPs into the digestive tract of organisms from the exposure group.

higher compared to the corresponding control ($P < 0.001$), while the activity measured in 21-days old individuals exposed to 12.5 μg/mL of PLA was significantly lower than the corresponding control ($P < 0.001$).

Although a significant effect of the exposure time was noted on lipid peroxidation levels ($F_{3,23} = 67.052$; $P < 0.001$; Fig. 3f), with an overall increase observed between 7- or 14-days old individuals and 21-days old ones ($P < 0.001$ in both the cases), no effect of the treatment ($F_{3,23} = 2.398$; $P = 0.094$) occurred. Moreover, a significant time × treatment interaction ($F_{6,23} = 6.433$; $P < 0.001$) was noted, but no biologically-relevant interactions were noted.

3.2. Responses of energetic biomarkers

The complete outcome of the statistical analyzes performed on data of energetic biomarkers is reported in Table S2. Independently of the treatment, a significant effect of the exposure time on protein ($F_{3,24} = 131.917$; $P < 0.001$), carbohydrate ($F_{3,22} = 346.07$; $P < 0.001$) and lipid content ($F_{3,24} = 530.74$; $P < 0.001$), as well as on the total energy content ($F_{3,24} = 419.30$; $P < 0.001$), was noted. For all the endpoints, levels measured in 7-days old individuals were lower than those from 14- ($P < 0.001$ for all the endpoints) and 21-days old conspecifics ($P < 0.001$ for all the endpoints). No significant effect of treatment ($F < 2.333$; $P > 0.100$ in all the cases) was noted for all the energetic biomarkers, independently of the exposure time. A significant time × treatment interactions was observed for all the energetic biomarkers ($F > 3.564$; $P < 0.011$ in all the cases), except for the carbohydrates content. Protein content in 7 days-old exposed to 0.125 μg/mL was higher than the corresponding control ($P < 0.001$; Fig. 4a), while for the same concentration the levels were lower in 14-days old individuals compared to corresponding control ($P < 0.001$; Fig. 4a). Moreover, the levels measured in 14-days old organisms exposed to 1.25 μg/mL and to 12.5 μg/mL of PLA-MPs were higher than the corresponding control for lipid ($P < 0.001$ for both the cases; Fig. 4c) and caloric content ($P = 0.016$ for 1.25 μg/mL and $P < 0.001$ for 12.5 μg/mL, respectively; Fig. 4d).

3.3. Swimming behavior

The complete outcome of the statistical analyzes performed on data of swimming behavior is reported in Table S3. Overall, the application of LMMs did not show significant effects of the treatment for both the considered endpoints. In detail, no effect of the treatment was found for the distance moved ($F_{3,8.00}= 2.817$; $P = 0.107$) and for the swimming speed ($F_{3,8.00}= 2.609$; $P = 0.123$). However, a significant effect of the time of exposure was noted for the distance moved ($F_{2,338.07}= 82.085$; $P < 0.001$) and swimming speed ($F_{2,337.10}= 104.211$; $P < 0.001$), with 14- and 21-days old individuals travelled a longer distance and swam quicker than 7-days old ones ($P < 0.001$ for the pairwise comparisons for both the endpoints). A significant time × treatment interaction occurred for the distance moved ($F_{6,338.07}= 3.264$; $P < 0.003$) and the swimming speed ($F_{6,337.10}= 3.879$; $P < 0.001$), with 21-days old individuals exposed to 12.5 μg/mL of PLA-MPs that travelled longer distances ($P < 0.001$; Fig. 5a) and swam quicker ($P < 0.001$; Fig. 5b) than conspecifics from the corresponding control.

4. Discussion

This work showed that neither short-term nor long-term exposure to PLA-MPs induce mortality in *D. magna* neonates or adults. However, a slight effect on the oxidative status, energy reserves and swimming behavior was observed in *D. magna* individuals.

Our results agreed previous studies showing that the ingestion of polyethylene (PE; size range 63–75 μm, 21 days of exposure to 25, 50, and 100 μg/mL; Canniff and Hoang, 2018) and polystyrene (PS; $Ø = 1$ μm and 10 μm, 21 days of exposure to 0.125, 1.25 and 12.5 mg/L; De Felice et al., 2019) microbeads did not induce mortality in individuals of the same species. However, they were not consistent with previous findings demonstrating that the exposure to MPs made of two bioplastic polymers, such as polybutylene adipate terephthalate (PBAT) and polyhydroxybutyrate (PHB), induced acute effects (i.e., mortality) on the same cladoceran species (Göttermann et al., 2024). The lack of acute effects observed in our study could be due to the limited ingestion of PLA-MPs by daphnids or adults. In fact, nevertheless the ingestion was confirmed with microscopy analysis the size of the most of administered PLA-MPs exceeded the size range of food that can be ingested by our model species, at least at its juvenile stages. Indeed, Gophen and Geller (1984) showed that *Daphnia* spp. Individuals usually ingest food particles ranging between 700 nm and 70 μm. However, previous studies pointed out that *D. magna* individuals were able to ingest polyethylene MPs in the 10–106 μm size range (Frydkjær et al., 2017) and up to 250 μm in size (Kokalj et al., 2018). However, stressful conditions might be induced not only by ingestion but also by the external interaction between PLA-MPs and *D. magna.* In fact, previous studies found MPs adhering on some external structures of both daphnids and adults, including the carapax and/or the appendages, which could affect the health status and behavior (e.g., De Felice et al., 2019).

In spite of the lack of acute effects, PLA-MPs exposure slightly modulate the oxidative status of adult individuals. Overall, our results did not show a statistically significant increase of ROS content (Fig. 3a), disagreeing some results from previous studies showing an increase in the ROS levels after the exposure to high concentrations of MPs (Cui et al., 2023). However, the modulation in antioxidant enzyme activity indirectly suggested that PLA-MPs exposure induce an alteration of the oxidative status of treated organisms, which were able to counteract the potential toxicity of pro-oxidant molecules. Indeed, despite a slight modulation of SOD activity, an overall activation of both CAT and GPx enzymes was noted in individuals exposed to the highest concentration of PLA-MPs compared to control conspecifics, independently of the time of exposure. Thus, the SOD modulation could indirectly suggest the production of hydrogen peroxide, whose toxicity was then counterbalanced by the activation of GPx and CAT, which play a complementary role in detoxification of hydrogen peroxide. Whilst CAT was activated only at the end of the exposure to the highest tested concentration, a significant increase in GPx was already noted after 14 days of the exposure to the highest tested concentration, as well as to the intermediate one (Fig. 3c). Additionally, a significant modulation in GST activity was observed in response to intermediate and high tested concentrations. The increase of the GST levels observed that the organisms up-regulated antioxidant defenses, which is also supported by literature (Klaper et al., 2009; Rodrigues et al., 2019). The decrease of activity measured after the exposure to 12.5 μg/mL might be due to the conjugation of glutathione (GSH) with electrophilic compounds operated by this enzyme to prevent toxic effects (Kim et al., 2010). Although our results did not evidence an overproduction of ROS levels the modulation in the oxidative status suggested the imbalance of the oxidative status, which can lead to an oxidative stress condition. The lack of the occurrence of oxidative damage, in terms of lipid peroxidation, suggested that organisms activated an efficient antioxidant machinery that prevented the toxic effects of pro-oxidant species generated by PLA-MPs exposure. These results support previous findings that identified MPs as mediators of alteration in oxidative status and induction of oxidative stress conditions. Indeed, the modulation of SOD, CAT and GPx activity was induced by the exposure to MPs with different size, shape and polymer composition, as well as in other aquatic model species (e.g., Jeong et al., 2016; Jeong et al., 2017; Lu et al., 2016; Murano et al., 2021; Magni et al., 2018). Similar modulations in antioxidant defenses were observed also in aquatic organisms exposed to PLA-MPs. For instance, a recent study performed on *D. magna* showed that chronic exposure to 1 and 5 mg/L of PLA-MPs resulted in different expression of the *sod* gene, with a down-regulation observed at 5 mg/L of PLA-MPs and an up-regulation at the lower concentration (An et al., 2024). These results suggested that at the high concentration organisms coped with oxidative stress, which resulted in the decrease of offspring, the increase of the sex ratio and

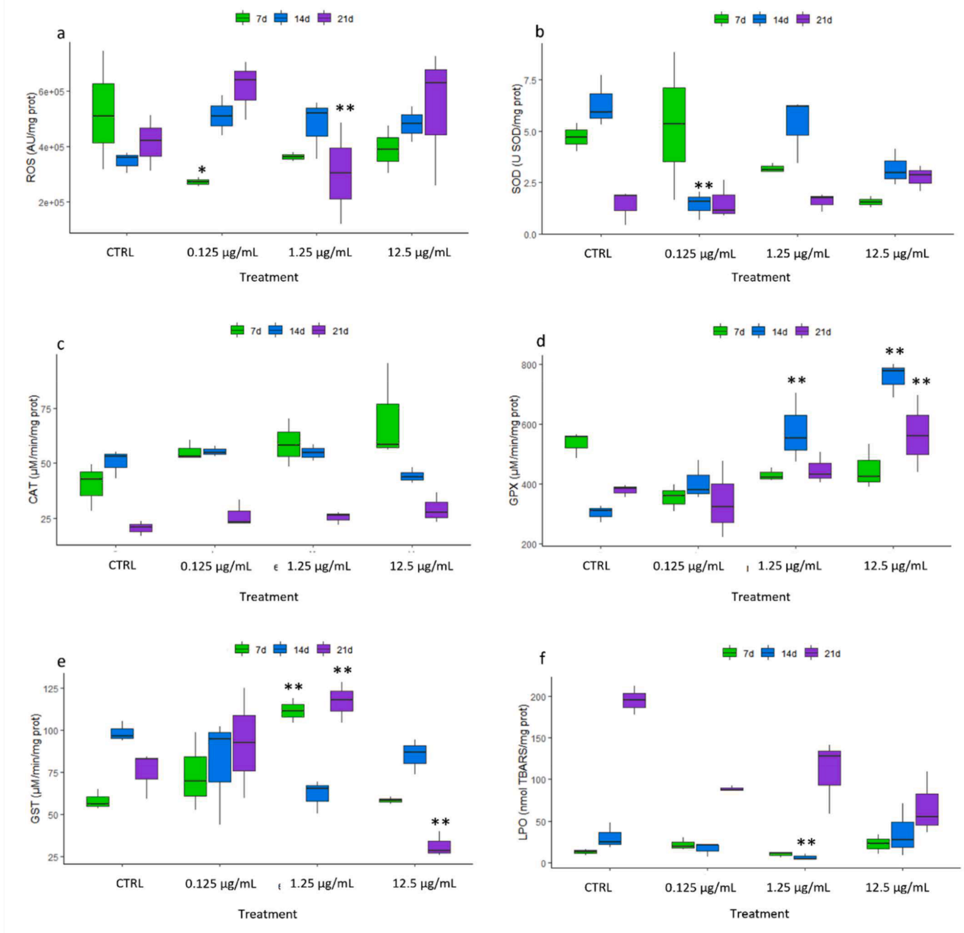

Fig. 3. Box plots of oxidative stress biomarkers measured in *Daphnia magna* individuals after 7, 14 and 21-days exposure to PLA-MPs. ROS (a) SOD (b), CAT (c), GPx (d), GST (e) and LPO (f). Asterisks above the box plots show significant differences between treated individuals and the corresponding control (* $P < 0.05$; ** $P < 0.01$).

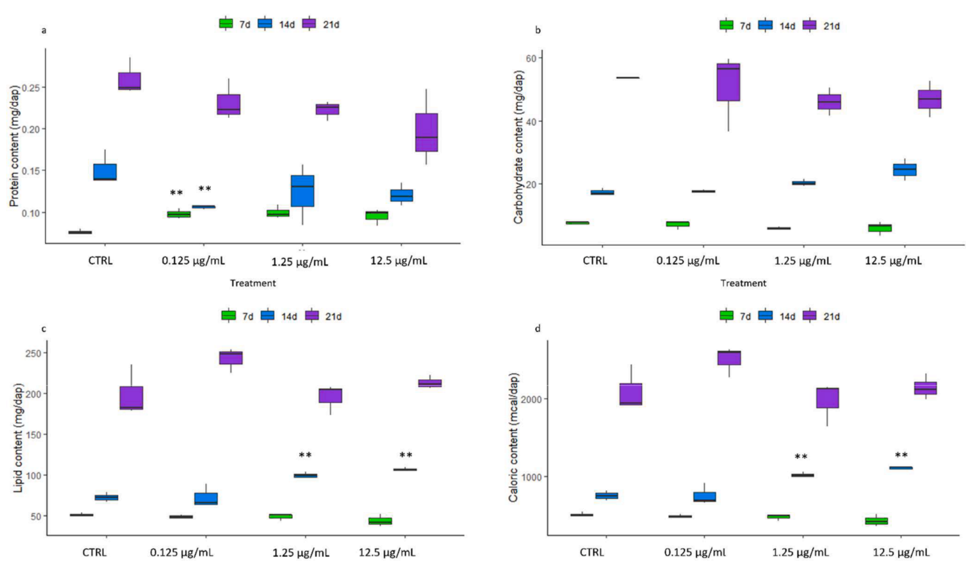

Fig. 4. Box plots of the energetic biomarker responses measured in *Daphnia magna* individuals after 7, 14 and 21-days of exposure to PLA-MPs. Protein content (a), carbohydrates content (b), lipid content (c) and caloric content (d). Asterisks above the box plots show significant differences between treated individuals and the corresponding control (* $P < 0.05$; ** $P < 0.01$).

deformed embryo, as well as high mortality (An et al., 2024). Moreover, antioxidant responses were also observed in the blue mussel *Mytilus edulis* exposed to PLA-MPs (10 – 100 mg/L; Khalid et al., 2021).

Organisms exposed to stressful conditions activate defense machinery and therefore they use energy, resulting in changes of the physiological status of the organism (Keum et al., 2010; Nagato et al., 2016). Several studies highlighted that the exposure to MPs can lead to the alteration of metabolomic profiles, such as lipid and carbohydrate contents (Tourinho et al., 2022; Parolini et al., 2023; Deng et al., 2017). Moreover, a recent study showed that the exposure to PLA-MPs induced the modulation in the expression of the arginine kinase (AK; An et al., 2024), an enzyme involved in cellular energy metabolism catalyzing the conversion of adenine nucleotides (Lyu et al., 2015; Huang et al., 2020). As a normal consequence of the physiological development and growth process of this species (Schür et al., 2020) a significant increase in energy reserves (i.e., protein, carbohydrate, lipid and total caloric content), was observed in *D. magna* individuals independently of the treatment. However, PLA-MPs exposure induced an increase, compared to the corresponding control, in protein content after 7 and 14 days of exposure to 0.125 μg/mL, as well as in both lipid and caloric content after 14 days of exposure to 1.25 μg/mL and 12.5 μg/mL (Fig. 4). The increase in protein content observed at the low concentration and early exposure time could be related to the synthesis of enzymes involved in the defense mechanisms (Gorokhova et al., 2013; Sytar et al., 2013). In fact, organisms can increase their energy reserves as a mechanism of defense against the exposure to contaminants (Sokolova et al., 2012). For instance, organism exposed to low concentrations of contaminants can use lipids and carbohydrates for metabolic purposes, while they increase protein content thorough the synthesis of defense enzymes (Smolders et al., 2002). Thus, the increase in lipid and caloric content observed in response to 1.25 and 12.5 μg/mL PLA-MPs could suggest that treated individuals accumulated energy reserves to meet the increasing energy demand for counteracting the toxicity of PLA-MPs. This hypothesis is supported by previous studies on freshwater invertebrates, which highlighted an increase in energy content after the exposure to a stressful condition (Sokolova et al., 2012) and to polystyrene microplastics (Parolini et al., 2023) or nanoplastics (De Felice et al., 2022). However, because of the limited knowledge concerning the role of contaminants in modulating the energy reserves in *D. magna*, an alternative explanation of our results could refer to a better assimilation of food in presence of PLA-MPs. Previous studies showed that MPs of different polymers can act as carriers of nutrients, favoring their intake and enhancing lipid accumulation (Guo et al., 2020). Lastly, the enhancement of energy reserves could be due to an increase in swimming activity; the exposure to PLA-MPs could promote swimming activity and, consequently, filtering activity and food intake. This hypothesis was supported by our data as an increase in swimming activity, in terms of distance moved and swimming speed, observed after 21 days of exposure to 12.5 μg/mL of PLA-MPs (Fig. 5). The enhancement of swimming activity could be referred to an avoidance behavior fulfilled by the organism to swim away from a contaminated environment (Lopes et al., 2009, Lopes et al., 2004; Lovern et al., 2007). Alternatively, the increase of swimming activity could be interpreted as an attempt by treated organisms to get rid of the MPs adhering to body surface and appendages, as supposed by a previous study on *D. magna* exposed to the same concentrations of polystyrene MPs (De Felice et al., 2019).

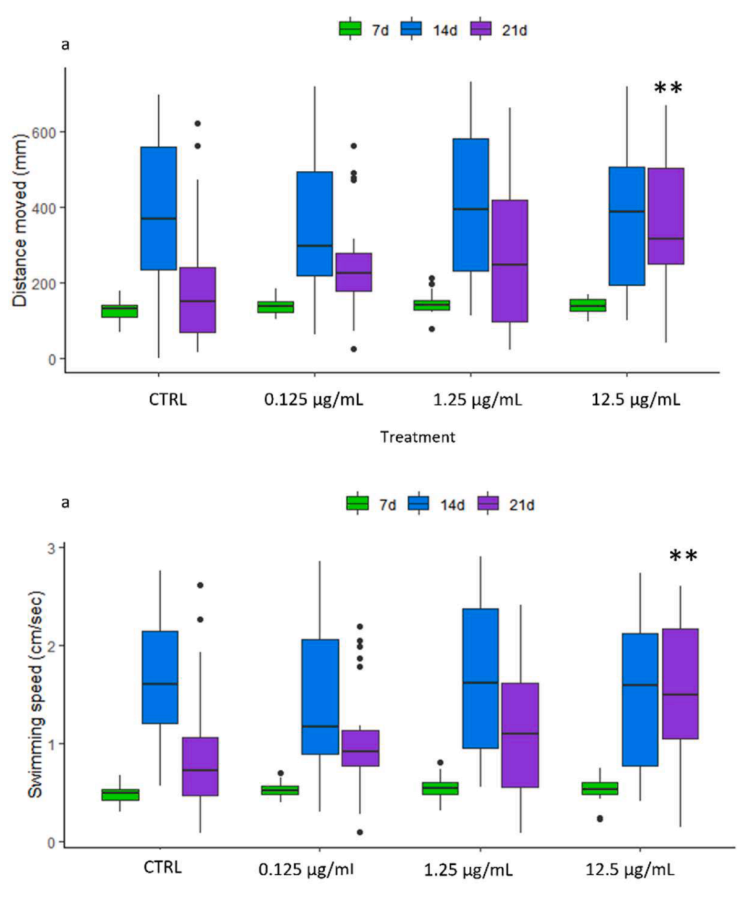

Fig. 5. Box plots of swimming behavior endpoints: distance moved (a) swimming speed (b), after 7, 14 and 21-days of exposure to PLA-MPs. Asterisks above the box plots show significant differences between treated individuals and the corresponding control (per each time point; * $P < 0.05$; ** $P < 0.01$).

5. Conclusions

The present study shows that the exposure to MPs derived from one of the most used bioplastic polymers could cause adverse effects to a freshwater model organism, in a similar way to MPs from conventional, fossil-based polymers. Although PLA-MPs did not induce significant acute toxicity, sub-lethal effects raised in treated individuals exposed to different concentration and time in relationship to the endpoint observed compared to controls. Changes in the oxidative status and slight energy imbalances were noted, mostly at the end of the exposure to 1.25 μg/mL and 12.5 μg/mL. A modulation in swimming activity was noted after the 21-days exposure to 12.5 μg/mL. Considering the predicted increasing use of bioplastics in our society and the subsequent potential increase in the environmental levels of MPs originated from the fragmentation and/or degradation of bioplastic products, there is an urgent need to test for toxicity of these contaminants. Therefore, further studies are necessary to investigate the properties of bio-based plastics and MPs, as well as their interactions with aquatic and terrestrial organisms. Moreover, as bioplastics can be degradable in the environment, it should be interesting to explore the degradation of MPs in the water, the release of degradation byproducts and their toxicity.

Consent to participate

Not applicable

Consent to publish

Not applicable

Ethical approval

Not applicable. As an invertebrate, the use of *D. magna* as a model organism does not need the approval by an appropriately constituted committee.

CRediT authorship contribution statement

Beatrice De Felice: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Stefano Gazzotti:** Investigation, Writing – review & editing. **Marco Aldo Ortenzi:** Investigation, Writing – review & editing. **Marco Parolini:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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